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ARTICLE

Functional Analysis of *Pcipg*2 From the Straminopilous Plant Pathogen *Phytophthora capsici*

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Summary: *Phytophthora capsici* causes serious diseases in numerous crop plants. Polygalacturonases (PGs) are cell wall-degrading enzymes that play an important role in pathogenesis in straminopilous pathogens. To understand PGs as they relate to the virulence of *P. capsici*, *Pcipg*2 was identified from a genomic library of a highly virulent *P. capsici* strain. *Pcipg*2 was strongly expressed during symptom development after the inoculation of pepper leaves with *P. capsici*. The wild protein (PCIPGII) was obtained from the expression of *pcipg*2 and found that increasing activity of PGs in PCIPGII-treated pepper leaves was consistent with increasing symptom development. *Asp* residues in active sites within *pcipg*2 affected PCIPGII activity or its virulence on pepper leaves. Results show that *pcipg*2 is an important gene among *pcipg* genes, and illustrate the benefit of analyzing mechanisms of pathogenicity during the period of host/parasite interaction. genesis 47:535–544, 2009. © 2009 Wiley-Liss, Inc.

Key words: *pcipg*2; straminopile pathogen; virulence; PGs activity; pathogenesis-related protein

INTRODUCTION

Many fungi and bacteria secrete remarkable arrays of plant cell wall-degrading enzymes (CWDEs) during interactions with plants, among which are polygalacturonases (PGs), pectin methylesterases (PMEs, EC 3.1.1.11), pectate lyases (PLs), and cellulases (Collmer and Keen, 1986). PLs can hydrolyze highly esterified pectins without the prior action of other enzymes (Alaña *et al*., 1989). PMEs remove the methyl ester groups present on the carboxyl moiety of galacturonic acid and produce methanol and acidic pectin for degradation by PGs and PLs in plant cell walls (Wu *et al*., 2005). PGs are the pectic enzymes that hydrolyze polygalacturonan, and are the key components of the pectinases.

PGs are further classified into endoPGs and exoPGs, although some enzymes exhibited both endo- and exo-PGs activities (Cooper *et al*., 1978). EndoPGs (EC 3.2.1.15) cleave the backbone of polygalacturonan internally, whereas exoPGs (EC 3.2.1.67) hydrolyze monomers progressively from the nonreducing end of the substrate. The endoPGs play an important role in fungal pathogenicity. Abundant evidence has confirmed that PGs are directly involved in the pathogenic processes of fungi (Garcia-Maceira *et al*., 2001; Shieh *et al*., 1997; Ten Have *et al*., 1998).

Mature PGs of plants and microbes share eight amino acid residues that are strictly conserved. These residues are 178N, 180D, 201D, 202D, 223H, 224G, 256R, and 258K (Van Santen *et al*., 1999). The amino acid residues of 180D, 201D, 202D, 223H, 256R, and 258K play an important role in enzyme activity, and three strictly conserved aspartic (*asp*) residues contribute to hydrolysis of glycosidic bonds and might be primary components in the activity site of most PGs (Armand *et al*., 2000; Pouderoyen *et al*., 2003).

Previous studies demonstrated that, similar to true fungi, straminopilous *Phytophthora* species secrete pectic enzymes in culture (Clarke, 1966; Yan and Liou, 2005; Yuan and Tseng, 1980), but knowledge of the molecular mechanisms of pectic enzymes secreted during growth and pathogenesis by *Phytophthora* species is limited and is not related to their economic and ecological importance. Recent studies have identified a few *pg* genes from *Phytophthora* species. *Pipg* 1 encoded an extracellular endoPG (Torto *et al*., 2002). In *P. cinna-*

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momi, one large *pg* gene family with 19 members was identified (Götesson *et al.*, 2002). Individual members of another *pg* gene family in *P. parasitica* have a specific role in decomposition of host plant cell wall (Wu *et al.*, 2008), and 3 *pg* genes (Genbank: DQ415987, DQ415988, EF558847) and 21 *Pg* genes of *Phytophthora capsici* were downloaded from the Joint Genome Institute (http://genome.jgi-psf.org/PhycaF7/PhycaF7.home.html). No data about *pg* gene functions related to pathogenicity factor in *P. capsici*.

P. capsici Leonian causes serious diseases in numerous crop plants (Kim and Hwang, 1992; Lamour and Hausbeck, 2002). *P. capsici* can deploy an array of host cell wall modifying enzymes during pathogenesis, and PGs are important parts of that array (Jia *et al.*, 2009). Although some *pg* genes have been identified from several *Phytophthora* species (Götesson *et al.*, 2002; Torto *et al.*, 2002; Tyler *et al.*, 2006; Wu *et al.*, 2008), the roles of PGs genes in the pathogenesis have not been conducted in *P. capsici.*

One of the main objectives of the present study was to investigate *pcipg*2 expression levels in pepper leaves inoculated with *P. capsici* using RNA blot analysis, followed by Western blot analysis to detect the levels of expression of the *pcipg*2 encoding protein in diseased leaves, based on the purified protein obtained from expression in Yeast *Pichia*. Site-directed mutagenesis was used to analyze the three strictly conserved *asp* residues in the active site within *pcipg*2, and the effect on wild protein activity and its virulence on pepper leaves. These experiments were designed to deduce whether *pcipg*2 is a major gene in cell wall degradation leading to development of lesions. We determined that *pcipg*2 participates in the infection process and in the development of Phytophthora blight in pepper.

RESULTS

PGs Activity of *P. capsici* SD33

A highly virulent strain of *P. capsici*, SD33, with high PGs activity was selected from more than 100 single-zoospore strains. The activity of a variety of PGs was detected in culture filtrates of SD33 daily for 13 days on OMP medium (Masago *et al.*, 1977). The PGs activity increased steadily from 1 to 7 days, reaching a maximum at the 7th day, then gradually decreased until the 13th day (see Fig. 1). These results suggest that PGs samples of SD33 from 1 to 7 days would be optimal for determining essential characteristics of the enzyme.

Isolation and Analysis of *pcipg*2 Structure

One complete sequence of a *pcipg* gene (*pcipg*2) was identified (GenBank accession numbers: DQ415987). A multiple sequence alignment of all the *pcipg* genes reveals *pcipg* 2 to have higher homology of 82.27% with a *pcipg* gene from JGI (jgi/phycaf7/20609) than with any other *pcipg* gene in genome sequences of *P. capsici*, but none was uniform to *pcipg*2. The open reading frame

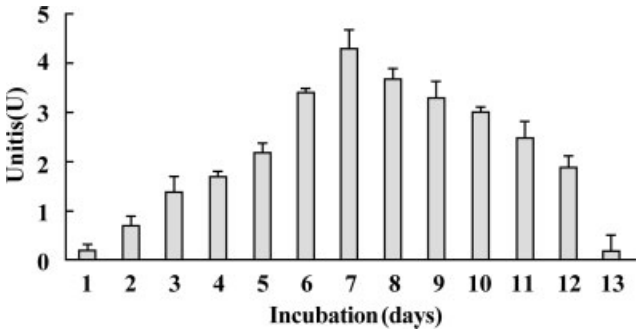

FIG. 1. PGs activity trends of *P. capsici* SD33 growing for 13 days on OM medium. PGs activity was assayed by determining the liberated reducing end products.

*pcipg*2 has 1,086 bp and encodes a polypeptide of 362 amino acid residues with a predicted molecular mass of 37 kDa. *Pcipg*2 contains a signal peptide of 19-amino acid residues and has three *N*-glycosylation sites, but does not exhibit an intron. On the basis of an alignment of most of the amino acid sequences of all reported PG genes, *pcipg*2 contains four highly conserved sequence segments in the catalytic site; and eight amino acid residues within these segments are highly conserved, and four of these highly conserved segments exist only in PGs of straminopilous pathogens (see Fig. 2). The numbers of these eight amino acid residue positions in *pcipg*2 are N^{177} XD^{179}, $D^{200}D^{201}$, $H^{222}G^{223}$, K^{260}, and R^{258}. *Pcipg*2 has two conserved residues (D^{182} and Y^{292}) adjacent to the active site, and they are involved in substrate binding in *Aspergillus niger* endoPGII (Pagès *et al.*, 2000). Three highly conserved amino acid residues of D^{179}, D^{200}, and D^{201} contributed to hydrolysis of glycosidic bonds in PGs of *A. niger* (Armand *et al.*, 2000; Pouderoyen *et al.*, 2003); they are also primary components of active site in PGs of *P. capsici*, and are identical to most other PGs. We further evaluated the role of *pcipg*2 in the degradation of cell walls and in pathogenicity during the infection of pepper plants by *P. capsici*.

The Expression of *pcipg*2, and Four Mutations and Purification

A protein (PCIPGII) with an apparent molecular weight of 40 kDa was expressed within 1–7 days after inoculation (Fig. 3a). The molecular weight of this protein was predicted to be 37 kDa, somewhat less than the actual weight. This difference is possibly due to the fact that *pcipg*2 has three *N*-glycosylation sites, which would influence the molecular weight of this protein when expressed by the yeast. Using an His Trap HP affinity column, the fusion proteins including PCIPGII, PCIPGII/179M, PCIPGII/200M, PCIPGII/201M, and PCIPGII/179M-200M-201M were easily purified to homogeneity, and each of these purified fusion proteins yielded a single band of about 40 kDa. The yield of each of the mutated proteins recovered was identical to that of PCIPGII (Fig. 3b). Additionally, the expression trend of

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1     ATGAAGACTTTTGGTACGCTCGTAGGTCTGTTACTTTTCGCTTTCACTGCGAACGGCTCG
1     M K T F G T L V G L L L F A F T A N G S

61    CCTATGCTGCGCGAAGGAACGGAGGCTGGAGACCCCTGCAACCTCACTGGAACTTACCAG
21     P M L R E G T E A G D P C N L T G T Y Q

121   AGTGGCACCGACATTTCATCGTGCAGTACCATCACGGTTGGGTCTCTTACGGTACCCGCT
41     S G T D I S S C S T I T V G S L T V P A

181   GGTGTCACGCTTGATCTGAGTAAGGCGAAGGACGGCGCTACCATCAACTTCACCGGTACG
61     G V T L D L S K A K D G A T I N F T G T

241   ACTACCTTCGGCACAGCAAAATGGGCTGGACCCCTCGTGTCGATCACCGGTAATTCCCTC
81     T T F G T A K W A G P L V S I T G N S L

301   ACCGTCAAGGGATCCGGCACGTTGGACGGTCAGGGCGACTGGTACTGGAAGCAGGGCGAG
101    T V K G S G T L D G Q G D W Y W K Q G E

361   TCGATCACTCGACCTGTCTTCGTGAAGCTGCAAAACGTCGTCGGTTCGAACATTTCGGGC
121    S I T R P V F V K L Q N V V G S N I S G

421   TTCACCGTGAAGAACTCTCCCTTTCGTACGTTTAGCGTCGTTACCTGTCAACAAACGACC
141    F T V K N S P F R T F S V V T C Q Q T T

481   ATCACTGGGCTCACGCTAGACTCCAGTGCTGGCAACGGCCTTGCTCAAAACACGGACGGC
161    I T G L T L D S S A G N G L A Q N T D G

541   TTCGATATGACCAAGAACGACCACGTGACGATCACTGGCAACAAGATTTACAACCAGGAC
181    F D M T K N D H V T I T G N K I Y N Q D

601   GACTGTCTTGCCATGCAGTCCAGTACGAACACCGTCTTCAGCAACAACTACTGCAGTGGC
201    D C L A M Q S S T N T V F S N N Y C S G

661   GGCCACGGCATCTCCATCGGATCGCTTGGTGGTTCCAGTGTGAGCTCAGCCGACACGGTG
221    G H G I S I G S L G G S S V S S A D T V

721   TCAGGCCTCACGGTCAAGGGCAACACCATCGTCAACAGCGTTAATGGTATCCGCATCAAG
241    S G L T V K G N T I V N S V N G I R I K

781   ACCATCGTGGATCTCAAGGGTCTTGTGTCCGACGTCACGTACACCGACAACAAGCTGAGC
261    T I V D L K G L V S D V T Y T D N K L S

841   AACGTCAAGAACGCCATCGTGATCCACTCGGACTACAGCAAGTCCAAGGGCGGATACACC
281    N V K N A I V I H S D Y S K S K G G Y T

901   GGTAAGGCTTCGAGCGCAGTGACCATTAAGGGCATCACCGTCTCGGGGCTCTCAGGTACG
301    G K A S S A V T I K G I T V S G L S G T

961   GCGACCAACCTGTACGACATCGTGGCCAACTCCAAGGTGGTGTCCAACTGGAAGTTCTCG
321    A T N L Y D I V A N S K V V S N W K F S

1021  GGCATCACCGTCAAGGCATCCAAGACGGGCAAGTGCAGCGGTCAGCCCAGCACAGTCAAG
341    G I T V K A S K T G K C S G Q P S T V K

1081  TGCTAA
361    C *
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FIG. 2. Comparison and alignment of deduced amino acid sequences of *Pcipg2*. Four highly conserved domains in all reported PGs are in black but not underlined. Four highly conserved domains that only exist in straminopilous pathogens are in black and underlined. Three aspartic acid residues (Asp179, Asp200, and Asp201) are regarded as the active-site components in most of the PGs are marked with an arrowhead. Three potential *N*-glycosylation sites are double underlined. Signal peptides are in boxes.

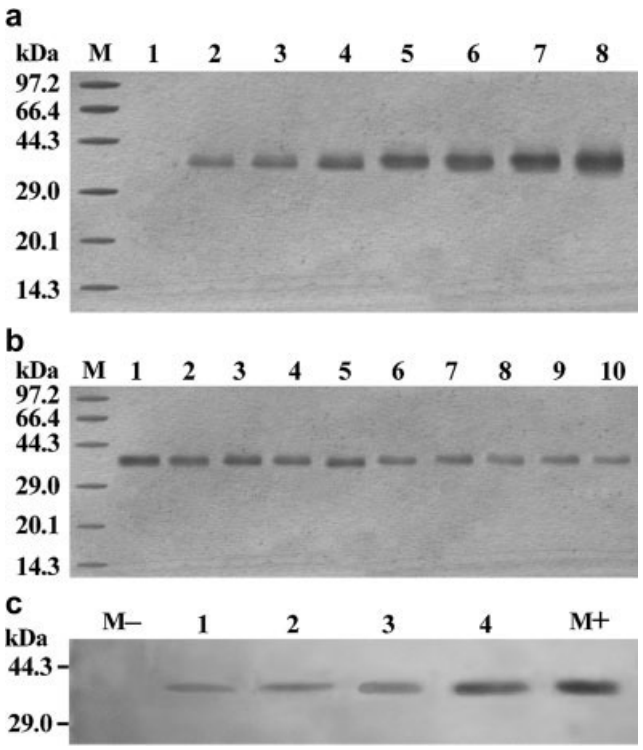

FIG. 3. (**a**) SDS-PAGE analysis of recombinant protein expression in *Pichia pastoris* GS115, lane 1: pPIC9k (empty vector), lanes 2–8: recombinant fusion protein expression from 1 to 7 days after induction. (**b**) SDS-PAGE analysis of purified fusion protein, lane 1: PCIPGII expression, lane 2: PCIPGII purification, lane 3: PCIPGII/179M expression, lane 4: PCIPGII/179M purification, lane 5: PCIPGII/200M expression, lane 6: PCIPGII/200M purification, lane 7: PCIPGII/201M expression, lane 8: PCIPGII/201M purification, lane 9: PCIPGII 179M-200M-201M expression, lane 10: PCIPGII 179M-200M-201M purification. M: low molecular weight marker. (**c**) Western blot analysis of PCIPGII expression level during *P. capsici* SD33 infected pepper leaves using antiserum. Lanes 1–4: Expression level of PCIPGII (40 kDa) at 1-day intervals from 1 to 7 dpi. M−: negative control. M+: positive control.

each mutated protein was similar to that of PCIPGII (Data not shown). Single mutations and simultaneous mutations in the sequence of *pcipg*2 are shown in the Supporting Information Data (Figs. 9–13).

Western Blot Analysis of *pcipg*2 Expression in Diseased Pepper Leaves

A single protein band with a specific electrophoretic mobility (40 kDa) was produced each day, and the band was always qualitatively equivalent to PCIPGII (Fig. 3c). This specific product in the treated leaves gradually increased in amount, and correlated with increased disease severity from 1 to 7 dpi. The protein quantity was low at 1 dpi, increased after 2 dpi (data not shown), and finally reached a maximum at 7 dpi. The protein expression level detected in the diseased pepper leaves at 7 dpi was nearly equivalent to the band present when the antibody reacted with the PCIPGII. This protein was present in the positive control and was absent in the negative control, and no additional protein bands were detected in the diseased leaves at various dpi. Thus, the expression of this specific protein was upregulated in a time-dependent manner after inoculation with SD33. These results suggest that the protein appearing in the diseased leaves is a specific product expressed by *pcipg*2 during infection by *P. capsici* SD33, and that PCIPGII plays a role in virulence on pepper leaves during infection by SD33. A similar result was obtained with BcPG 1 purified from *Botrytis cinerea* (Poinssot *et al.*, 2003).

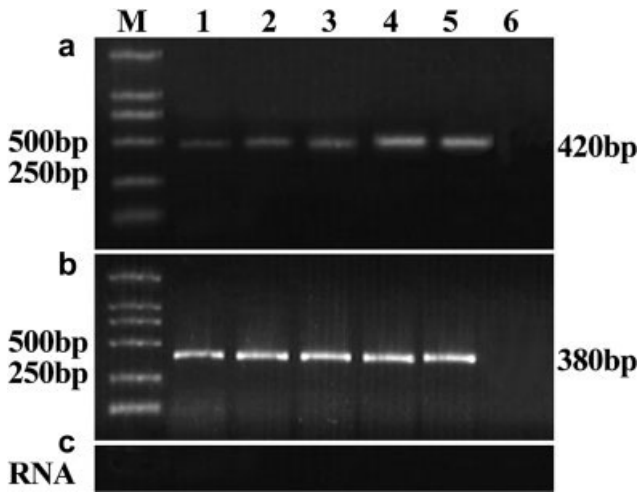

FIG. 4. RT-PCR analysis expression of *pcipg2* after SD33 infection of pepper leaves. (**a**) Lanes 1–4, cDNA from diseased leaves at 1-day intervals from 1 to 7 dpi, lane 5; cDNA from SD33; lane 6, cDNA from fresh pepper. (**b**) β-Actin used as a loading control. (**c**) Lane 1, RNA from fresh pepper leaves; lanes 2–5, RNA from diseased leaves at 1-day intervals from 1 to 7 dpi; lane 6, RNA from SD33. M: molecular standard. The entire experiment was conducted three times with similar results.

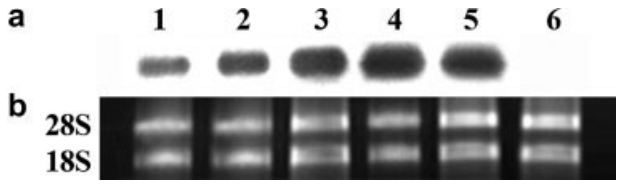

FIG. 5. (**a**) Northern blot analysis of *pcipg2* expression in infected pepper leaves, lanes 1–4: RNA from infected leaves at 1-day intervals from 1 to 7 dpi; lane 5: RNA of SD33 used as a positive control; lane 6: RNA of fresh pepper leaves used as a negative control. (**b**) 28S rRNA and 18S rRNA are also shown (lower panel).

RT-PCR and Northern Blot Analysis of *pcipg2* Expression on Diseased Pepper Leaves

Pepper leaves inoculated with zoospore suspensions of SD33 exhibited increasing severity of symptoms from 1 to 7 dpi. Small water-soaked lesions appeared on the leaves at 1 dpi, followed by the appearance of small dark lesion spots at 2 dpi, and more visible lesions from 3 to 5 dpi. The necrotic lesions expanded gradually and eventually resulted in partial leaf maceration around the inoculation sites at 7 dpi. *Pcipg2* transcription levels gradually increased from 1 to 7 dpi. Also, *Pcipg2* expression levels increased only slightly from 3 to 5 dpi, and lower levels observed at 1 and 2 dpi. *Pcipg2* expression levels were visibly induced at 7 dpi, producing an amplified fragment corresponding to that of cDNA from SD33 was observed (Fig. 4a). The absence of an amplified product in RT-negative reactions excluded DNA contamination. Similar results were also obtained from Northern blot analysis (see Fig. 5). These results suggest that *pcipg2* is differentially expressed in diseased leaves at various dpi, and that as expression levels increased over 7 days, lesion size increased correspondingly. Thus, *pcipg2* is hypothesized to encode a basic pathogenesis-related protein that was strongly induced during SD33 infection of pepper leaves.

Development of Disease Symptoms and PGs Activity in Pepper Leaves Treated With PCIPGII and Four Mutated Proteins

The presence of lesions in the positive control leaves was regarded as a typical disease symptom compared with that in the treated leaves. The treated leaves began to display small necrotic spots at the third day after treatment (dat) with PCIPGII, while they did not exhibit disease symptoms at 1 dat, and only exhibited small water-soaked regions at 2 dat. Disease assessment was conducted from 1 to 7 dat. In contrast to the typical symptoms developed in positive control leaves, necrotic lesions developed slowly in leaves treated with PCIPGII. Visible necrotic lesions appeared after 5 dat (Fig. 6a), but were not typical of Phytophthora foliar blight symptoms (Fig. 6f). The mean lesion area in leaves treated with PCIPGII was 0.25 cm^2, distinctly smaller than that of the positive control (2.5 cm^2) in 14 treated pepper leaves (see Fig. 7). The mean lesion area in the positive control was significantly different from that of leaves treated with PCIPGII ($P < 0.05$) (data not shown). However, the disease symptoms and the mean lesion area in leaves treated with PCIPGII were clearly distinguished from those of leaves treated with four mutated proteins and negative controls, respectively (Figs. 6b–e,g,h, and 7). Therefore, PCIPGII could induce necrotic lesions on the pepper leaves, resulting in pathogenic symptoms.

PGs activity was low at 1 dat in leaves treated with PCIPGII, and then began to increase at 2 dat (see Fig. 8). This observation is consistent with the slight symptoms (water-soaked regions) that appeared on the leaves following inoculation with PCIPGII from 1 to 2 dat. PGs activity then gradually increased and eventually showed definite activity peaks at 4 dat. Moreover, the necrotic lesion severity in leaves treated with PCIPGII gradually increased, in parallel with PGs activity from 1 to 7 dat (see Fig. 8). In addition, PGs activity in the positive control was higher than that in leaves treated with PCIPGII from 1 to 7 dat. For example, the maximum value of PGs activity in leaves treated with PCIPGII was always lower than that in the positive control leaves, and PGs activity peaks in the positive control continued up to 7 dat. These results provided a feasible explanation for why the area of necrotic lesions in positive controls was larger than that in the PCIPGII-treated leaves. It is notable that PGs activity was low in leaves treated with four mutated proteins, heat-killed protein and the negative control, which was consistent with the absence of symptoms or no lesion spots in the treatment pepper leaves. The conclusion from these results is that the development of disease symptoms in the treated pepper leaves was closely related to PGs activity.

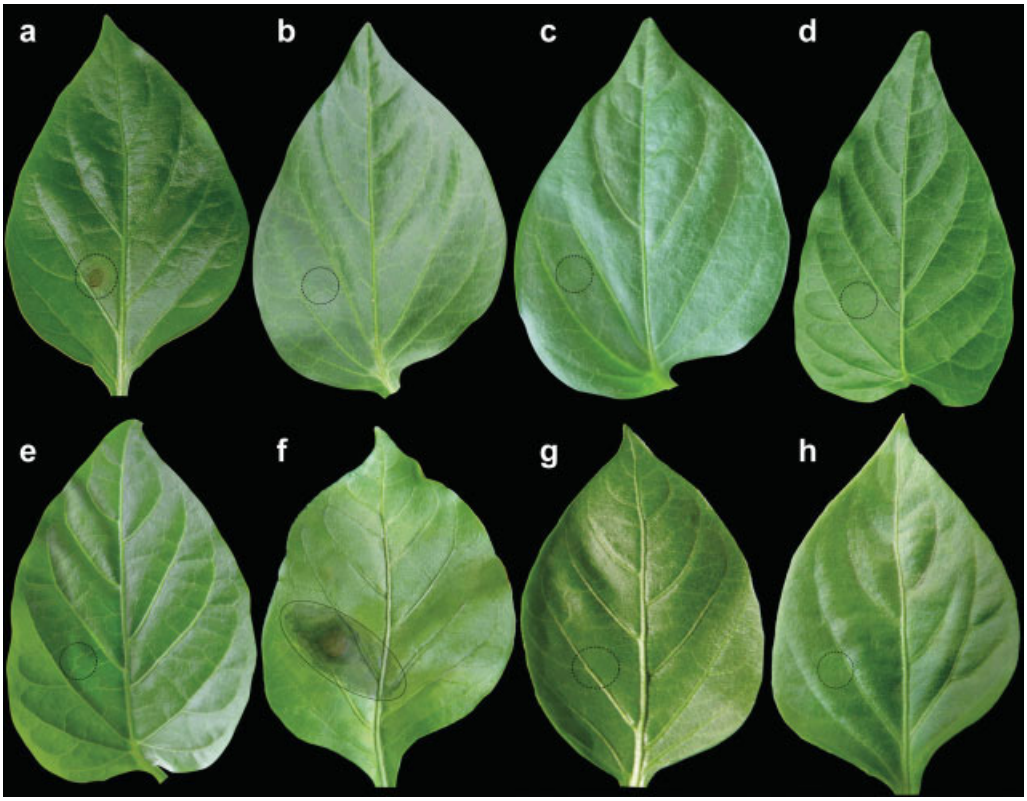

FIG. 6. Symptoms appearing on *Capsicum annuum* L. leaves after inoculation with PCIPGII, four mutated proteins, zoospore suspensions (positive control), heat-killed protein and distilled water (negative control). Equal volumes of 2.5 μl were injected. Zones with lesions are marked with a dotted black circle. Necrotic lesions were present in the leaves treated with PCIPGII, and any symptom did not appear after treatment with four mutated proteins, heat-killed protein, and distilled water, respectively. Pictures were taken 5 days after treatment. The letters **a, b, c, d, e, f, g** and **h** are PCIPGII, PCIPGII/179M, PCIPGII/200M, PCIPGII/201M, PCIPGII/179M-200M-201M, zoospore suspensions, heat-killed protein, and distilled water, respectively.

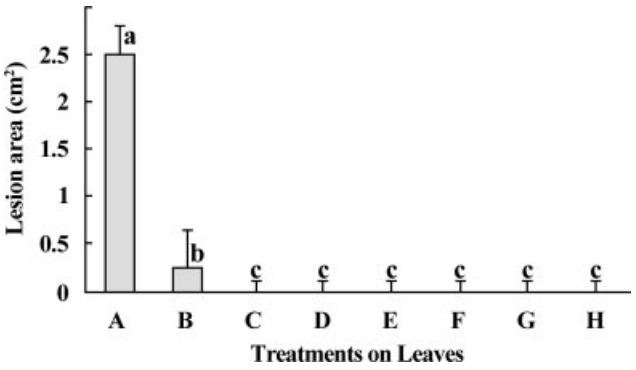

FIG. 7. Bars represent the mean ± standard error of 14 leaves. A: zoospore suspensions, B: PCIPGII, C–F: four mutated proteins respectively, G: heat-killed protein, H: distilled water. Lower case letters in common were not different significantly according to Fisher's protected least significant difference test ($P < 0.05$). The mean lesion areas were evaluated and photographed at 5 dat.

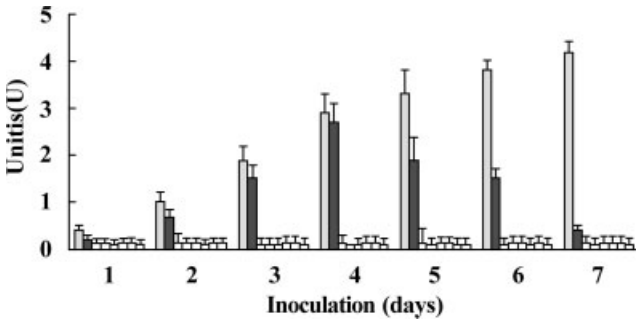

FIG. 8. PGs activity trends in crude protein extracted from pepper leaves infiltrated with single constructs or combinations from 1 to 7 days after inoculation with PCIPGII, four mutated proteins, zoospore suspensions (positive control), heat-killed protein and distilled water (negative control). Each column group consists of zoospore suspensions (bias bar), PCIPGII (black bar), and six short bars represent four mutated proteins, heat-killed protein, and distilled water, respectively. PGs activity was assayed by determining the liberated reducing end products.

DISCUSSION

The results of the present study showed that *pcipg2* exhibited similar expression trends at various dpi in Northern blot, RT-PCR, and Western blot analyses (Figs. 3c, 4, and 5). Notably, the production detected by each of these three methods correlated with the development

of disease symptom in the treated leaves at various dpi. Moreover, *pcipg*2 expression was strongly upregulated during infection of pepper plants by *P. capsici*. Furthermore, Western blot detection suggested that the pathogenesis-related protein (PCIPGII) encoded by *pcipg*2 might be in an active state during the course of symptom development, and its function was further validated by the production of disease symptoms in leaves treated with PCIPGII. *Pcipg*2 also encoded a basic pathogenesis-related protein that was strongly induced during infection by *P. capsici*, suggesting that the development of disease symptoms might involve the triggering of *pcipg*2 as a pathogenesis-related response in the infected leaves. The data show that *pcipg*2 transcription levels increased during infection, and *pcipg*2 expression was strongly upregulated during the interaction of *P. capsici* with pepper leaves. We speculate that other *pcipg* genes in this gene family might also express and secrete pathogenicity-related proteins during infection by *P. capsici*. We suggested that other *pcipg* genes in the host–pathogen interaction might effectively express and secrete virulence proteins during infection of pepper plants by *P. capsici*.

The PGs was encoded by many *pcipg* genes after inoculation pepper leaves with zoospore suspensions, while the PGs was only encoded by *pcipg*2 after the treatment of pepper leaves with PCIPGII. The zoospores could continuously produce a series of CWDEs such as PGs during the interaction with pepper leaves, whereas the PGs could not be produced in leaves treated with PCIPGII. This may be the actual reason why PGs activity levels in the positive control was always higher than that in leaves treated with PCIPGII at various dpi (see Fig. 8). The PGs activity trend in the leaves treated with PCIPGII or with the zoospore suspensions was correlated with development of disease. Conversely, PGs activity was considerably lower in pepper leaves treated with four mutated proteins and correspondingly no symptoms were produced (Figs. 8 and 6b–e). Similar results were found with the mutation of Pg genes from *Botrytis cinerea* and *Claviceps purpurea* (Oeser *et al*., 2002; Poinssot *et al.*, 2003). Hence, we conclude that the loss of the mutated protein activity should be correlated with the replacement of essential *asp* residues in the active sites in *pcipg*2. Simultaneous mutations and the single mutations led to a dramatic decrease in the specific activity. It is proposed that the mutation of one of these three *asp* residues could lead to an inactive enzyme, thus, each of these three *asp* residues is involved in the activity of PCIPGII. Taken together, it is reasonable to expect that these three *asp* residues might be main components in active sites in most of the PGs of *Phytophthora* spp. These results provide insight into the role of other *pg* genes from other straminopilous pathogens, based on site-directed mutagenesis of conserved *asp* residues in the active site.

The appearance of disease symptoms on pepper leaves inoculated with zoospore suspensions was rapid and the lesions were strictly localized, a characteristic of the response of pepper leaves to *P. capsici* pathogen infection of a susceptible cultivar (García-Pérez *et al.*, 1998). The pathogenicity of *P. capsici* might also involve the production of pathogenesis-related enzymes (Sanchez *et al.*, 1994). Our results confirm that PCIPGII has high affinity and specificity, and is likely to soften pepper tissues and even cause death of plant cells, but in a way unlike that caused by zoospore suspensions (Fig. 6a,f). Thus, PCIPGII might be an important pathogenicity factor during infection of pepper plants by *P. capsici*.

The blight lesions visible at 7 dpi on positive control leaves were so severe that they could not be illustrated; therefore, the more easily visible lesions formed at 5 dpi were used to compare with those of PCIPGII, and four mutated proteins treatments individually (Fig. 6a,f). Symptom development in the leaves inoculated with zoospore suspensions progressed over a 7-day period, which might be due to other pathogenicity factors such as infection structures, signal genes, other CWDEs, and host-specific toxin are involved in the later stages of symptom development (Idnurm and Howlett, 2001; Kronstad *et al*., 1998; Shea and Poeta, 2006; Stone *et al*., 2000). Some kind of CWDEs or host-specific toxins produced by plant pathogens frequently induced the death of the host cells (Stone *et al*., 2000). It has previously been suggested that *P. infestans*, *P. parasitica*, and *P. sojae* could produce CWDEs to initiate death of host cells (Takemoto *et al.*, 1999; Yan and Liou, 2005). Although it has also been suggested that *P. capsici* could produce pathogenicity factors such as CWDEs, little data have been reported. PCIPGII appears to induce plant cell death in areas surrounding the inoculation site, resulting in lesions. Increasing evidence suggests that *pcipg*2 plays an important role in pathogenicity of *P. capsici*, and one may speculate that PCIPGII could represent an excellent novel agent for degrading plant cell walls or damaging plant tissues. Results suggest that (i) *pcipg*2 regulates the induction of this enzyme, which is required for virulence during *P. capsici* infection of pepper; (ii) the mutant proteins revealed almost no infection on the pepper hosts, which suggests that the damage of this enzyme is related to its activity; (iii) *pcipg*2 may be an important pathogenicity gene in the *pcipg* gene family members of *P. capsici*, and other *pcipg* genes may embody nonexclusive virulence functions in this gene family. Although gene-for-gene interactions are currently unknown in *Phytophthora*-pepper/other hosts system, our results clearly indicated that we should explore the potential functions of *pcipg*2 as a virulent gene on pepper hosts.

MATERIALS AND METHODS

Strain SD33, PGs Activity Assay, and Construction of *P. capsici* Genomic Library

Strains of *P. capsici* were isolated from blighted, diseased pepper plants, and identified as *P. capsici* as described by Waterhouse (1963). To induce *P. capsici*

strain to produce PGs, each strain was incubated on OMP medium (1.25% oat, 1% pectin, pH 7.0) at 28°C for 13 days. The virulence of all strains was determined by Sun *et al*. (2008), and their PGs activity was detected by the protocol of Jia *et al*. (2009).

A genomic DNA from SD33 was extracted as previously described (Panabieres *et al*., 1989). The library was constructed using pUC19 as the vector, after digestion with *Bam*HI. After separation by sucrose density gradient centrifugation, DNA fragments with sizes ranging from 2.5 to 4.0 kb were collected by ethanol precipitation. Ligation of the DNA with pUC19 vector, and transfection of the plasmid DNAs into *Escherichia coli* strain DH5α were performed according to instructions provided by the manufacturer. Colonies were randomly selected from the library and subjected to DNA sequence analysis for analyzing the inserts. The titer of the original genomic library was estimated to be 1.5×10^5 colonies.

Isolation of *pcipg*2 and Sequence Analysis

The genomic library was screened using two pairs of degenerate primers: P710(5′-ACGG(A/C/G/T)CA(A/G)GG(A/C/T)GC(T/C)T(A/G)(G/T)TACTGG-3′)/P1150R(5′-GATGATGGTCTTGATGCGGA-3′), which were designed based on the conserved sequences of other *pg* genes (Götesson *et al*., 2002; Torto *et al*., 2002; Tyler *et al*., 2006; Wu *et al*., 2008; Yan and Liou, 2005). *Pcipg* genes were screened from the genomic library according to the procedure described, and minor adjustments made to the PCR reaction systems and parameters (Liu *et al*., 2000). Five microliters sample of the reaction mixture was examined by 1.2% agarose gel electrophoresis to check the products. Genomic DNA was used as positive control and water was used as negative control. A single clone containing the *pg* gene was determined and sequenced using an Applied Biosystems 3730 DNA Analyzer (Foster City, CA). The plasmid clones were sequenced using universal primers M13F and M13R. To verify the *pg* gene amino acid sequence, sequence data were analyzed using appropriate programs in the GCG software package (Genetics Computer Group, Wisconsin Package Version 10.0). Nucleotide and amino acid sequence homology searches were compared with the sequences in the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/). Most of available complete *pg* amino acid sequences including straminopilous pathogens, fungi, plants, and bacteria were multialigned using Clustal X 1.83 (Thompson *et al*., 1997) and GeneDoc (version 2.6.002) (Nicholas *et al*., 1997). The PG sequences of *P. ramorum* and *P. sojae* were downloaded from the US Department of Energy Joint Genome Institute (http://www.jgj.doe.gov/).

Expression of *pcipg*2 in *Pichia pastoris* and Purification

*Pcipg*2 was expressed by using an EasySelect *Pichia* Expression Kit (Invitrogen, Carlsbad, CA 92008). The *pcipg*2 cDNA was used as template for PCR to obtain a DNA fragment corresponding to the predicted *pcipg*2 of amino acid residues 20-362. Primers of *pcipg*2-GSP1(5′-ACTCGAATTCCACCACCACCACCACCACGACG ACGACGACAAGTCGCCTATGCTGCGCG-3′) (affixation C-terminal His_6 tag) and *pcipg*2-GSP2 (5′-ACTTG CGGCCGCTTAGCACTTGACTGT-3′). All procedures for expression and purification were similar to those described previously (Shi *et al*., 2007). The PCR parameters were as follows: 94°C for 4 min, 35 cycles of 94°C for 1 min, 62°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were cloned into the pGEMT-easy vector to generate the pGEMT/*Pcipg*2 plasmid. The *pcipg*2 was excised as an *EcoR* I/*Not* I fragment from pGEMT-*pcipg*2 and then subcloned into pPIC9K with a C-terminal His_6 tag to generate the expression vector pPIC9K/*pcipg*2, which was then transformed into *Pichia pastoris* GS115 strain using a *Pichia* EasyComp transformation kit (Invitrogen). Transformants (GS-pPIC9K/*pcipg*2) were selected on plates with YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar) and G418 (4 mg/ml). To induce the recombinant protein to express sufficiently, the transformants were inoculated in BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.04% biotin, 1% glycerol, and 4 mg/ml G418) and grown in shake culture (250–300 rpm) at 30°C for 24 h. The culture was then resuspended in BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 13.4% yeast nitrogen base without amino acids, 0.04% biotin, 0.3 mM sodium molybdate, and 0.5% methanol) and incubated for 7 days. The supernatant was recovered by centrifugation at 10,000*g* for 10 min at 4°C for each of the 1- to 7-day-old cultures, and then the protein examined by SDS-PAGE. The empty vector pPIC9K was used as a control.

After incubation for 7 days, the protein was purified from culture supernatants using a HisTrap HP column (Amersham Biosciences, Uppsala, Sweden) and was named PCIPGII. A 5-ml column was filled with Ni Sepharose medium and equilibrated with binding buffer (20 mM Na_3PO_4, 500 mM NaCl, 5 mM imidazole, pH 7.4). The sample was applied to the pre-equilibrated column, washed with binding buffer, and the recombinant fusion protein was eluted by a linear gradient of 5–500 mM imidazole in buffer (20 mM Na_3PO_4, 500 mM NaCl, pH 7.4) at 1 ml/min. Both the flow-through and the eluted fractions were collected and analyzed by 12% SDS-PAGE and stained with Coomassie brilliant blue R-250 (Bio-Rad, Foster City, CA). The protein elution profiles were monitored at 280 nm, and its activity was determined as previously described.

Site-Directed Mutagenesis of *pcipg*2, Expression, and Purification

Based on the alignment of all *pg* genes as previously described and all *pg* genes from JGI, three *asp* residues (179D, 200D, and 201D) are presumed to be the puta-

tive active site in *pcipg*2. Active-site-directed mutagenesis was used to further investigate *pcipg*2 functions. The single mutations and simultaneous mutations were performed by overlap PCR (Gote *et al*., 2007; Horton and Pease, 1991). The primers used for mutation are A (5′-GCCTGAATTCCACCACCACC-3′) and B (5′-CTCTGCGG CCGCTTAGCAC-3′) for flanking the complete *pcipg*2, had *Eco*R1 and *Not*1 restriction sites, respectively, in their 5′ ends to give the desired orientation when ligated into the expression vector. The internal overlapping primers are C (5′-CAAAACACGGAAGGCTTCG-3′) and D (5′-CGAAGCCTTCCGTGTTTTG-3′) for (179D-D179E), E (5′-GACAGTCTTCCTGGTTG-3′) and F (5′-CAACCAGGAA GACTGTC-3′) for (200D-D200E), G (5′-CAAGACAGTTG TCCTGG-3′) and H (5′-CCAGGACAACTGTCTTG-3′) for (201D-D201N). Two separate PCRs were carried out on the template to give products of AC and DB. The products have the mutation incorporated at their ends. The final product AB was made by hybridizing the overlap strands from the two fragments and extending this overlap with DNA polymerase. For single mutant, two separate PCRs were carried out using the recombinant plasmid pPIC9K/*pcipg*2 as the template. To obtain the products of AC and DB, PCR reactions systems and parameters as described previously (Obradors *et al*., 1998) with minor adjustments. The PCR products were purified and then mixed. Overlap extension was performed using the flanking primers to amplify the recombinant products as well as the nonproductive strands. The conditions for the overlap PCR were similar to the first PCR reaction except equivalent DNA of products AC and DB were used as template. The products of the overlap extension with a C-terminal His_6 tag were purified, digested with *Eco*RI and *Not* I, and ligated into the expression vector pPIC9K previously digested with the same restriction endonuclease. The procedure to obtaining D200E and D201N was similar to that of D179E. Three plasmids (pPIC9K/179M*pcipg*2, pPIC9K/200M*pcipg*2, and pPIC9K/201M*pcipg*2) were obtained. Each plasmid was sequenced to verify the desired substitutions introduced and to confirm the absence of additional changes in the coding sequence. The simultaneous mutation was performed by procedures similar to those of the single mutation. The pPIC9K/179M*pcipg*2 was used as the template, and pPIC9K/179M-200M*pcipg*2 was obtained and then sequenced to verify the double mutation succeeded. The pPIC9K/179M-200M*pcipg*2 was then used as the template for D179E/D200E/D201N, and pPIC9K/179M-200M-201M*pcipg*2 was obtained and then sequenced to verify the mutation succeeded. Four mutated proteins were obtained according to the expression and purification procedure of the wild protein, and they were then designated PCIPGII/179M, PCIPGII/200M, PCIPGII/201M, and PCIPGII/179M-200M-201M, respectively. The empty vector pPIC9K served as a negative control, and the wild protein served as a positive control. The activity of four mutated proteins was determined using the previously described methods.

Preparation of PCIPGII Antibodies and Western Blot Analysis

PCIPGII was used to prepare antibodies in New Zealand white rabbits according to standard protocols (Kothari *et al*., 2006). The antibody preparation, purification, and antiserum titer determination were done as described previously (Yang *et al*., 2007). Zoospore suspensions of SD33 were used as inoculum to obtain diseased leaves of a highly susceptible cultivar Kexing 3, as described previously (Sun *et al*., 2008). Diseased leaves were collected at 1-day intervals from 1 to 7 days postinoculation (dpi), and the crude protein of the various treatment leaves was extracted by the method (Joubert *et al*., 2007). The crude protein samples were run on 12% SDS-PAGE gel, and then transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in TBS buffer (10 mM Tris base, 100 mM NaCl) for 1 h at 22°C before incubating with 1 μg/ml antibody (anti-*pcipg*2) in TBS for 16 h at 4°C. The membrane was then washed four times with TBS-T (TBS containing 0.1% Tween 20) for 5 min at room temperature, then incubated with 0.1 μg/ml of HRP-conjugated goat anti-rabbit antibody (Dako Cytomation, Glostrup, Denmark) in TBS buffer for 1 h. Antibody binding was detected by washing the membrane four times with TBS-T, and incubated with peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's recommendations. The PCIPGII was used as positive control, and the crude protein from fresh leaves inoculated with sterile distilled water was used as negative control. Signals were detected by enhancing chemiluminescence (Cell Signaling Technology, Beverly, MA).

RT-PCR and Northern Blot Analysis of *pcipg*2 Expression in Leaves Treatment With Zoospore Suspension

The pepper cultivar Kexing 3 was selected for inoculation with zoospore suspensions of strain SD33. Seedlings were grown as described previously (Sun *et al*., 2008). Detached leaves obtained during the fifth to sixth-leaf stage were placed in Petri dishes containing 1.5% (w/v) water agar and kept in dark for 7 days at 28°C after inoculation of each leaf with 2.5 μl of the zoospore suspensions (1×10^5 zoospores/ml). The production of sporangia and zoospores was induced by Sun *et al*. (2008). Control pepper leaves were inoculated with distilled water.

To extract total RNA, pepper leaves with necrotic lesions collected at 1-day intervals from 1 to 7 dpi were ground in liquid nitrogen. Total RNA was extracted using an RNeasy plant minikit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendation. Total RNA was used for reverse transcription by Omniscript RT kit (Qiagen). Synthesized cDNA was used for PCR (Fujikawa *et al*., 2006), and primers of RT-PF (5′-GGCACGTTG GACGGTCAGGG-3′) and RT-PR (5′-GCTGTTGAC GATGGTGTTGCC-3′) were designed from nonconservative regions of pcipg 2. β-Actin was used as an internal

control, the specific primers of β-actin 1 (5′-CTGGGACGACATGGAGAAGATC-3′) and β-actin 2 (5′-CGCTCCGTCAGGATCTTCATC-3′). The PCR amplification parameters were minor adjusted as described previously (Fonseca *et al*., 2005). The PCR products were loaded on 1.0% agarose gel electrophoresis and visualized by ethidium bromide fluorescence. Results were obtained from three repeated experiments. cDNA from SD33 was used as positive control. cDNA from fresh leaves and RNA from fresh pepper leaves were used as negative control. Northern blot was performed according to procedures (Sambrook *et al*., 1989), in which a nonconserved fragment was amplified using primers of RT-RF and RT-PR, and then used to synthesize a probe using the DIG DNA labeling kit from Roche following the manufacturer's protocol. RNA of SD33 served as positive control and RNA of fresh pepper leaves served as a negative control.

Treatment of Pepper Leaves With PCIPGII and Mutated Proteins

Before inoculation, seedlings of Kexing 3 were maintained at 100% humidity at 28°C for 24 h to maximize leaf opening during the fifth to sixth-leaf stage. PCIPGII, and four mutated proteins supernatant solutions were individually dialyzed twice at 4°C for 24 h against 50 volumes of distilled water to filter out ions. Quantitative analysis of each protein was carried out as described by Bradford (1976). To evaluate the impact of the different proteins on leaves, the seedlings were spot inoculated with 2.5 μl (0.7 μg/μl) of each protein solution using a microsyringe. Inoculations were carried out on three of the uppermost leaves. A second set of leaves inoculated with zoospore suspensions (1×10^5/ml) served as a positive control, and the heat-killed protein and distilled water served as a negative control. Following inoculation, seedlings were maintained at 100% humidity at 28°C for 7 days. The development of necrotic lesions on the leaves was observed daily for 7 days. The percentage of leaf area with lesions was determined using Image J software (Developed at the U. S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Each treatment was repeated at least three times. Data were analyzed by analysis of variance and Fisher's least significant difference test ($P < 0.05$) (Statview v. 5. 0.1: SAS Institute, Cary, NC). To determine PGs activity in pepper leaves treated with different proteins, abundant leaves were collected daily from 1 to 7 dpi, and crude protein was extracted. The PGs activity was determined as described above.

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